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THE DEFINITION OF EPSTEIN BARR VIRUS (EBV)'S ROLE IN HTLV III INFECTED USAF PERSONNEL AS RELATED TO DISEASE PROGRESSION

ANNUAL REPORT

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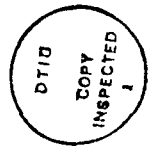
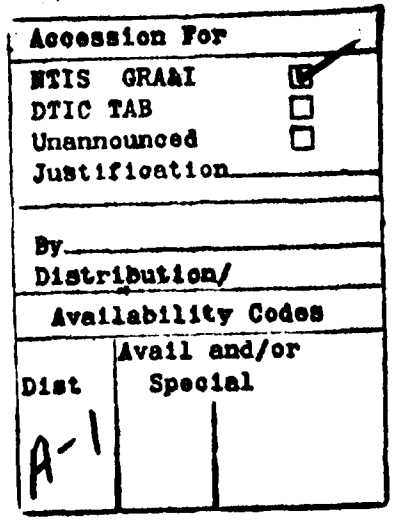
Foreword

For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administration Practices Supplements.





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Statement of Problem

Infection with human immunodeficiency virus type 1 (HIV-1), the cause of acquired immunodeficiency syndrome (AIDS), may remain quiescent for long periods, including years, without producing any clinical signs of disease. The present study hypothesizes that repeated reactivation of latent infection by strains of Epstein-Barr virus (EBV), a member of the herpesvirus group, and possibly other forms of *in vivo* interaction between HIV-1 and EBV provokes clinical progression of the HIV-1 infection. Abnormalities in immunoregulation of the latent EBV infection in patients with HIV-1 infections also can result in the production of severe lymphoproliferative lesions, lymphomas. or other as yet ill-defined manifestations.

Background

Although HIV-1 is essential to the development of AIDS, there is increasing evidence showing that EBV plays a significant role in the clinical progression of AIDS, producing manifestations that may be severe and fatal.(1) Earlier results have shown that patients with AIDS-related complex (ARC) and AIDS have an exaggerated antibody response to EBV antigens and a highly increased load of EBV in oropharyngeal secretions and peripheral mononuclear cells. (2) These findings by the principal investigator's laboratory indicated that there is recurrent reactivation of the lifelong latent EBV infection in patients with ARC and AIDS. B cell lymphomas containing EBV-DNA are being reported in increased frequency in homosexuals and AIDS patients, particularly now that these individuals are living longer through the support of antiretroviral chemotherapy.(3) The EBV-related tongue lesion, oral hairy leukoplakia, may be a poor prognostic sign when found in HIV infected patients.(4) EBV related lymphocytic interstitial pneumonia may produce significant morbidity and mortality in children with HIV infections.(5)

Approach to Problem (AIMS)

The aims of the current protocol were to determine if changes in EBV serologic findings and the quantity or type of EBV strains in oropharyngeal secretions and peripheral blood mononuclear cells correlated with clinical and immunologic progression of the underlying HIV-1 infection. As patients in this prospective study evolved into an increasingly more progressive HIV infection state, it was to be determined if EBV (or unusual strains of EBV) were present in selected pathologic lesions and also if abnormalities in T cell immunoregulation of EBV coincided with the progression event(s).

Materials and Methods

Study group. Individuals eligible to enroll in this study were required to have a documented HIV-1 infection as determined by the screening HIV-1 antibody program instituted by the Armed Services. Those individuals that were Air Force personnel were then evaluated for their HIV-1 infection at Wilford Hall Medical Center, San Antonio, Texas, and asked to sign the consent form for entry into the collaborative prospective study directed by Dr. (Col.) R. Neal Boswell and which includes this protocol. With the on site evaluation, the HIV-1 infection of the participants was then staged according to the Armed Forces (Walter Reed or WR) Disease Classification (Table 1). (6) Detailed EBV serologic and virologic studies for the present protocol were to be performed serially, every 6-12 months routinely, and, if possible, at time of clinical changes.

EBV Serologic Testing. Specific EBV serologic testing included antibody determinations to EBV capsid antigen (to include IgM, IgG, and IgA antibodies), diffuse and restricted components of EBV early antigen, and to EBV nuclear antigen. This testing involves standard indirect immunofluorescent techniques that have been described by the principal investigator and other workers. (2, 7)

EBV Virologic Testing. This testing included the following procedures: prevalence and titer of infectious EBV in oropharyngeal secretions according to a transformation assay, (8) quantity of EBV-DNA by spot hybridization, (9) and prevalence of EBV antigens utilizing reference sera or monoclonal EBV antibodies and a spontaneous proliferation assay (10) in peripheral blood lymphocytes and pathologic tissues.

Isolates of EBV were examined for strain differences by performing blot hybridization with cloned probes on agarose gel-separated products of extracted DNA that has undergone restriction enzyme digestions. (11)

EBV Immunologic Testing. The regression assay to be used to determine the efficiency of the patient's T cells in inhibiting the transformation of EBV infected B cells has been described by Crawford et al. (12)

Results

Serologic Findings. Serum IgM antibodies to EBV capsid antigen were not found in this relative small number of patients examined serologically during year 2 of the study. (Table 2). The prevalence of serum IgG antibody to

EBV capsid antigen was similar among specimens from patients in the individual WR classes and controls; the geometric mean titers (GMT) were also similar among specimens from patients in all the WR classes but in all cases were greater than that found in healthy controls. (Control rates in Table 4)

The prevalence and GMT of serum IgG antibody to EBV early antigen components was similar among the WR classes and uniformly greater compared to healthy controls (Table 2). The antibody response shifted from a predominantly anti-R to a response directed about equally to either D or R components as the WR classification advanced.

While the prevalence of antibodies to EBV nuclear antigen was similar among the WR classes (and controls), there was a trend to a relative decline in the GMT of this antibody response with advanced WR stages (Table 2).

Virologic Findings. The high positive rates of EBV in oropharyngeal secretions of patients with HIV-1 infections, compared to healthy controls, are relatively similar throughout the WR classes (Table 3). (Control rates are shown in Table 5)

In contrast, the high positive rates of peripheral blood spontaneous lymphoproliferation induced by EBV in patients with HIV-1 infections, compared to healthy controls, tend to decline (with the statistical constraints of the small number of higher WR classes examined) in those with more advanced WR classes, 5 and 6 (Table 3). Somewhat of a similar finding occurred with the increased estimated mean number of EBV-infected lymphocytes from peripheral blood specimens (Table 3). Healthy controls have been found to have <15% prevalence of spontaneous proliferation of peripheral blood lymphocytes and a mean count of <2 of EBV infected cells per 10^7 mononuclear cells.

Immunologic (EBV-specific) Findings. A regression assay examining predominantly the host's T8 cell immune capacity to restrict transformation revealed that a significant proportion of small number of patients in early WR classes tested to date had abnormal responses compared to healthy controls (Figure 1). The few patients tested so far preclude any analysis of interclass differences.

Patients with Oral Hairy Leukoplakia (OHL). Twenty-four HIV-1 infected males (almost all declared homosexuals or bisexuals) and one HIV-1 infected female were identified as having OHL. Twelve of these patients were in WR class 1. With rare exceptions, all OHL+ lesions contained herpesvirus-

like particles visualized by electron microscopy performed at Wilford Hall Medical Center.

As a group the OHL+ patients had greater titers of antibodies to EBV antigens of the replicative cycle, capsid antigen and early antigen, while more depressed titers of antibodies to EBV nuclear antigen than healthy controls (Table 4). The titers of antibody to capsid antigen in the OHL+ group were considerably greater than those noted in patients with acute EBV induced infectious mononucleosis (IM), while the antibody levels to early antigen were increased but to a lesser degree. In a comparison of the OHL+ group with WR 1 classification versus those with the same WR classification but lacking OHL (OHL-), the OHL+ group had more divergent anti-EBV capsid antigen and anti-EBV nuclear antigen responses compared to healthy controls.

The prevalence of EBV in oropharyngeal secretions in both the OHL+ and OHL- groups was greater than the rates seen in normal healthy controls while lower than IM controls (Table 5).

The rate of spontaneous lymphoproliferation of peripheral blood mononuclear cells in the OHL+ WR 1 group approximated the rate seen in the IM group and was greater than the OHL- WR 1 group (Table 5). The healthy control group had a considerably lower positive rate than all the other groups. The OHL+ WR 1 group had a greater estimated number of EBV infected cells in the peripheral blood compared to OHL- WR 1 patients, the former group having a virocyte count approaching that seen in acute EBV IM.

The titers of transforming EBV in oropharyngeal secretions from 9 OHL+ patients (Table 6) were considerably higher than levels noted in patients tested with acute EBV induced IM (mean/log 10 titer/ml 1.4) and in healthy controls that are excreting virus in these secretions (mean/log 10 titer/ml <0.6). Due to small numbers tested, it is not clear if this viral titer in oropharyngeal secretions in OHL+ patients was higher than that found in OHL- WR1 patients.

Cryostat sections of 10 tongue biopsies from OHL+ patients were examined for the presence of viral capsid antigen and early antigen by an indirect immunofluorescent technique using monoclonal antibodies to these specific antigens. EBV capsid antigen and early antigens were detected in 8 biopsies while viral capsid antigen alone was detected in one. Transforming EBV was grown from 2 of 10 OHL+ biopsy specimens placed in <u>vitro</u> cultivation with umbilical cord mononuclear cells.

Discussion

The presence of an intense broad spectrum response to EBV antigens starting early in the HIV-1 infection suggests that reactivated type EBV infections are particularly prominent in the initial stages of the underlying HIV-1 infection. Data from the first year of the study documented clearly that these intense antibody responses and those of a reactivated and not primary EBV infection, including those with the presence of IgM antibody to EBV capsid antigen. The inverse correlation between the intensity of the antibody response to EBV nuclear antigen and the shifting of antibody response from a response predominantly to the restricted (R) component to the diffuse (D) component of EBV early antigen with advanced WR classes also indicates that the host immune response to EBV remains quite intense and abnormal in comparison to normal healthy individuals.

These serologic findings are reflective of the abnormally elevated burden of transforming EBV present in oropharyngeal secretions and peripheral blood lymphocytes of patients with HIV-1 infections of all WR classes. Interestingly with advanced WR classes the level of EBV in peripheral blood lymphocytes declined, although still remaining above levels seen in healthy normal individuals while the amount of EBV in oropharyngeal secretions remained at stable elevated levels. It appeared then that patients with advanced HIV-1 infections have a tendency in continuing to produce high levels of replicating virus in oropharyngeal secretions while maintaining less elevated levels of virus in the latent form in peripheral blood lymphocytes in later stages of their HIV-1 infections. Further analysis of the actual titer of virus and EBV DNA present in these body fluids/secretions will assist in clarifying this unexpected phenomenon. The high levels of EBV present in patients with HIV-1 infections pose a significant problem because of the increased possibility that a clone with malignant potential could emerge and lead to a lymphoproliferative and thereafter lymphomatous process.

The subset of HIV-1 infected patients with OHL can provide important information on the potentially deleterious effects that may be produced by an overly large burden of EBV permitted by the host's abnormal immunoregulation of EBV. These patients appear to having a most intense level of EBV in peripheral blood lymphocytes and possibly oropharyngeal secretions in comparison to those HIV-1 patients of the same WR class. It remains to be shown if the extremely large numbers of EBV particles present in these lesions consists of any unusual DNA fragment patterns. However, it has been clearly shown that replicating virus is present in these tongue lesions. It is currently controversial if the

presence of OHL indicates a poor prognosis for HIV-1 infected patients. This point will be critically evaluated in the present prospective study.

The number of HIV infected patients with serial specimens and also with documented progression of their WR classification are much to few to determine if there are any EBV findings that correlate with clinical and immunologic progression of the HIV-1 infection. The data accumulated thus far provides a prevalence and cross-sectional evaluation of EBV findings in the study population. The continuation of this prospective study will allow for a better interpretation and extension of the present findings and correlation of EBV with progression of the HIV-1 infection and EBV related pathologic lesions.

Table 1

Armed Forces (Walter Reed Of WR)
Disease Classification of Infection
by Human Immunodeficiency Virus (HIV)

Class	HIV ANTIBODY and/or VIRUS ISOLATION	CHRONIC LYMPH-ADENOPATHY	T HELPER CELLS/mm3	DELAYED HYPER SENSITIVITY	THRUSH	OPPORTUNISTIC INFECTION
1	+	-	>400	Normal	-	-
2	+	-	>400	Normal	-	-
3	+	+/-	<400	Normal	-	-
4	+	+/-	<400	Partial anergy	-	-
5	+	+/-	<400	Partial/ complete anergy	+	-
6	+	+/-	<400	Partial/ complete anergy	+/-	+

Table 2

The prevalence and geometric-mean titer (GMT) of serum Antibodies to Epstein-Barr Virus (EB according to the Walter Reed (WR) classification.

	Class					
	1	2	3	4	5	6
IgM-CA						
Prevalence*	0/10(0)	0/31(0)	0/19(0)	0/11(0)	0/9(0)	0/8(0)
GMT	2.5	2.5	2.5	2.5	2.5	2.5
IgG-CA						
Prevalence	10/10(100)	31/31(100)	19/19(100)	11/11(100)	9/9(100)	8/8(100)
GMT	393.8	572.0	398.2	600.6	690.9	452.4
IgG-EA						
Prevalence+	9/10(90)	29/31(93.5)	17/19(89.5)	9/11(81.8)	8/9(88.9)	8/8(100)
Anti-D¥	1(11.1)	1(3.4)	9(52.9)	3(33.3)	3(37.5)	4(50.0)
Anti-R	7(77.8)	20(69.0)	6(35.3)	5(55.6)	4(50.0)	4(50.0)
Anti-DR	1(11.1)	8(27.6)	2(11.8)	1(11.1)	1(12.5)	0(0)
GMT+	34.8	47.8	37.2	42.6	34.3	36.7
Anti-EBNA						
Prevalence	7/7(100)	23/24(95.8)	18/19(94.7)	10/11(90.9)	9/9(100)	7/8(87.5)
GMT	20.0	26.7	38.6	15.5	27.2	14.1

* Number of positive specimens/total number of specimens (%)

\+ The prevalence and GMT of total positive reactions to EA components.

¥ The positive reactions were divided into these directed to the diffuse (D) component or restricted (R) component of EBV early antigen comples or an undifferentiated (DR) response. % are those of the positive reaction group.

Table 3

The prevalence and titers of EBV in oropharyngeal washings and peripheral blood mononuclear cells for HIV infected patients according to WR class.

	1	2	3	4	5	6
Oropharyngeal Washings						
Prevalence*	10/13	10/12	5/8	6/10	7/9	4/9
%	76.9	83.3	62.5	60	77.8	44.4
Peripheral mononuclear cells						
Prevalence of spontaneous proliferation	81/136	78/111	9/17	10/14	6/12	4/10
	59.6	70.3	52.9	71.4	50.0	40.0
Mean estimate+ (±1 SD)	63.1 (238.7)	342.8 (1389.1)	73.9 (234.8)	1.3 (3.3)	503.2 (761.6)	2 (4)

* no. of positive specimens/total no. of specimens. All the oropharyngeal washings have not yet been processed.

\+ no. of EBV infected cells per 10^7 mononuclear cells (±1 SD)

Table 4

Comparison Of Antibodies To EBV Antigens In Patients With OHL (OHL+) And In Three Control Groups

Antibodies	HIV-Infected: OHL+ (WR 1-6) (n=25)	HIV-Infected: OHL+ (WR 1 only) (n=12)	HIV-Infected: OHL-# (WR 1 only) (n=30)	Healthy Controls (n=31)	Controls with infectious mononucleosis (n=27)
IgM to EBV capsid antigen					
Prevalence	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	26 (96.4)
GMT	2.5	2.5	2.5	2.5	111.4
IgG to EBV capsid antigen					
Prevalence	22/22 (100)	11/11 (100)	28/28 (100)	30 (96.8)	27 (100.0)
GMT	497.2	466.9	275.8	136.4	288.9
IgG to EBV early antigen					
Prevalence	19/22 .86.4)	10/11 (90.9)	23/27 (85.2)	7 (22.6)	27 (100.0)
GMT	24.9	25.7	23.4	6.5	86.5
To EBV nuclear antigen					
Prevalence	20/22 (90.9)	11/11 (100)	25/25 (100)	30 (96.8)	10 (37.0)*
GMT	12.5	16.6	23.0	26.8	1.7

NOTE: Prevalence data are no. of patients (%). GMT = geometric mean titer. To figure this mean, we defined nondetectable titers for antibodies to EBV nuclear antigen (<2.5) and for all others (<10) as 1.25 and 5, respectively. Results of few sera are pending.

Age and Sex Matched

* The sera of these 10 patients had only low titers of antibody, 2.5-5.

Table 5

The Prevalence And Content Of EBV In Saliva And
Peripheral Blood Mononuclear Cells Of Patients With OHL And Controls

HIV-Infected

Parameter	OHL± (WR 1 - 6)	OHL± (WR 1 only)	No OHL-* (WR 1)	Healthy Controls	Controls with infectious mononucleosis
Oropharyngeal EBV	14/20 (70)	6/10 (60)	19/30 (63)	5/23 (21.7)	17/19 (89.5)
Spontaneous lympho-proliferation in vitro	11/23 (47.8)	7/11 (63.6)	13/30 (43.3)	3/23 (12.0)	10/15 (66.7)
Estimate of EBV-infected cells	86.6 (±289.0) (n=22)	149.5 (±379.0) (n=12)	17.93 (±56.8) (n=29)	1.9 (± 3.9) (n=7)	244.7 (±611.7) (n=9)

* Age and Sex matched

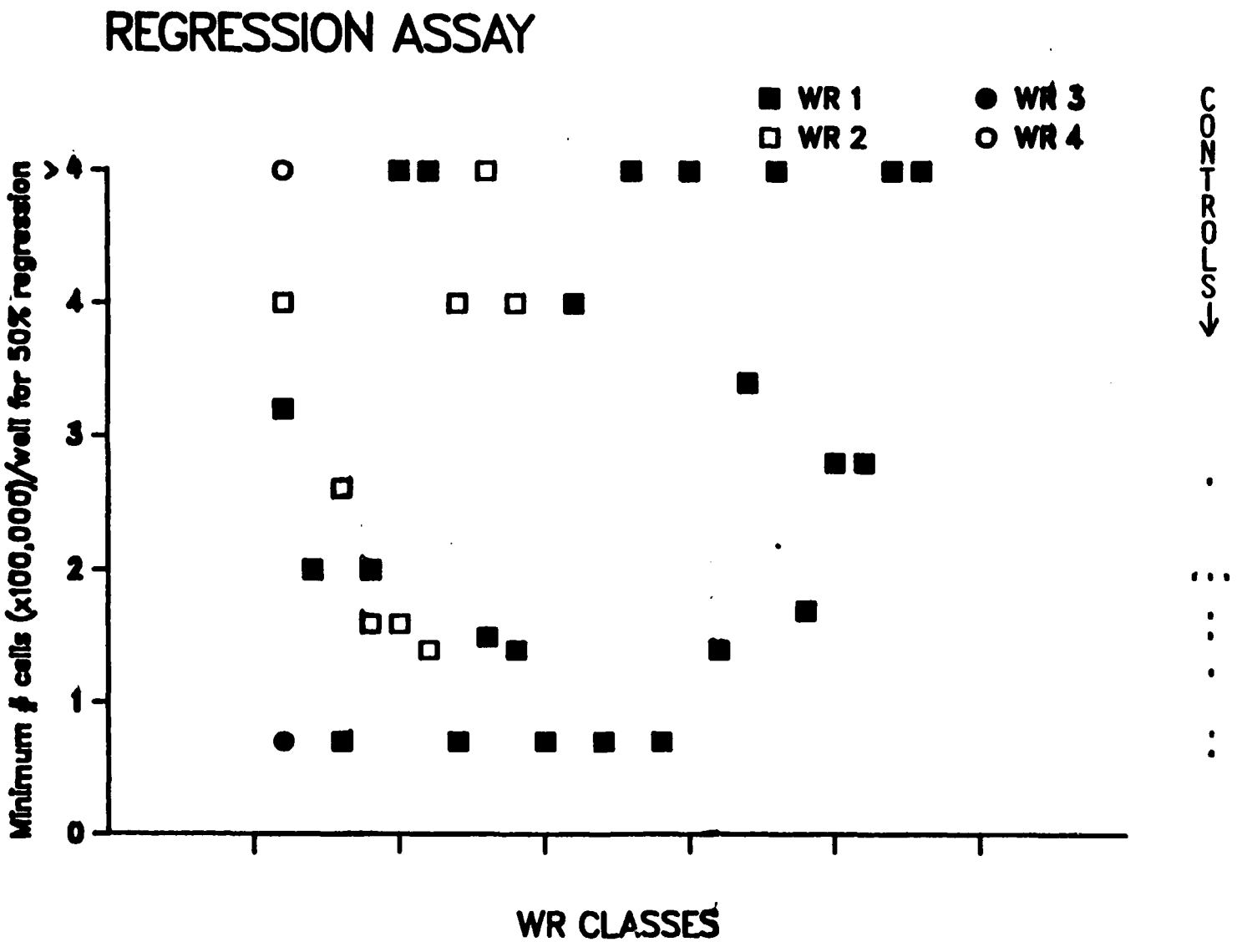
Note: Results are no. positive/no. tested (%), except for EBV-infected cells, which shows the no. of cells/10^7 mononuclear cells (± 1 SD).

Table 6

Titers Of Transforming EBV In Oropharyngeal Washings From 9 OHL Patients

Patient By WR Class	Titer (log_{10} TD_{50}/ml)
1A	4.3
1A	<1.0
1A	<1.0
1A	1.6
2A	2.2
4A	3.3
4A	2.9
5A	3.1
5A	3.5

FIGURE 1


REGRESSION ASSAY
WR 1
WR 2
WR 3
WR 4
CONTROLS
Minimum # cells (x100,000)/well for 50% regression
>4
4
3
2
1
0
WR CLASSES

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